Treating Genetic Disorders Using State-of-the-Art Technology

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Abstract

CRISPR/Cas9 [clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9], basically a bacterial immune system, is now widely applicable to engineer genomes of a number of cells and organisms because of its simplicity and robustness. In research avenue the system has been optimized to regulate gene expression, modify epigenome and edit target locus. These applications make CRISPR/Cas9 a technology of choice to edit disease causing mutations as well as the epigenome more efficiently than ever before. Meanwhile its application in *in vivo* and *ex vivo* cells is encouraging the scientific community for more vigorous gene therapy and in clinical setups for therapeutic genome editing. Here we review the recent advances that CRISPR/Cas9 mediated genome editing has achieved and is reported in previous studies and address the challenges associated with it.

Introduction

Genome editing has a profound consequence on disease treatment. In order to understand the function of gene in the disease pathway, the modulation of its expression remains the basic aim of classical genetics and modern molecular biology (Barrangou *et al.*, 2015). The development of RNA interference technology in the early 1990s and its application in mammalian cells to unveil the molecular functions of genes gave rise to the era of reverse genetics. The research area was further strengthened by the development of genome editing tools based on DNA-binding nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like nucleases (TALENs), and the recently discovered CRISPR/Cas9 system (Joung and Sander, 2013; Urnov *et al.*, 2010). The engineering of DNA binding proteins to target specific DNA sequences is time consuming and expensive. However, the simple design and high efficiency of CRISPR/Cas9 made it a famous genome editing tool, enabling genome editing in a number of organisms (Cho *et al.*, 2013; Ding *et al.*, 2013; Niu *et al.*, 2014; Wang *et al.*, 2013).

CRISPR/Cas9

CRISPR (clustered, regularly interspaced, short palindromic repeat)/Cas (CRISPR-associated protein) is an array of repeat and spacer sequences that work with Cas9 to protect bacteria and archaea against the invading genome (Wiedenheft *et al.*, 2012). About 90% of archaea and 40% of the sequenced bacterial genomes contain a CRISPR

system (Deveau *et al.*, 2008; Koonin and Makarova, 2009). Initially the system was identified by a Japanese research group in 1987; these repeat sequences were present in downstream of Iap gene (Ishino *et al.*, 1987). There are five different classes of CRISPR system among which Type II-A CRISPR system of *Streptococcus pyogenes* is well elaborated for its genome editing purpose. Naturally present Type II CRISPR/Cas9 system in *S. pyogenes* comprises a repeat-spacer-repeat sequence, which is transcribed into crRNA (CRISPR-RNA), the crRNA hybridizes with tracrRNA (trans-activating CrRNA) encoded by set of nucleotides present near the CRISPR locus. The hybridization of crRNA-tracrRNA known as guide RNA makes a ribonucleoprotein complex with Cas9 for invading DNA degradation (Doudna and Charpentier, 2014). In 2012, researchers used CRISPR/Cas9 from *S. pyogenes* for genome editing purposes (Jinek *et al.*, 2012). Cas9, after cleavage of double stranded DNA (dsDNA), activates the cell repair pathways to repair the dsDNA. These pathways include the erroneous pathways known as NHEJ (non-homologous end joining) which causes insertion or deletion mutations. In the presence of donor DNA (HDR template) the dsDNA is repaired by process known as HDR (homology directed repair) which causes knock-in/-out of gene of interest (Barnes, 2001; Dudás and Chovanec, 2004).

CRISPR/Cas9 has been applied both *in vivo* and *ex vivo* to treat the cells in individuals suffering from various genetic disorders, also optimized to generate disease model organisms (Table 3.1). To demonstrate the proof-of-concept for CRISPR/Cas9 as a therapeutic toolkit we summarize the ground breaking approaches carried out *ex vivo* and *in vivo* to treat the genetic flaws. Regarding genome editing, the delivery of Cas9–gRNA complex into the target organ or organ system can use different methods such as lipid mediated delivery, electroporation, cell penetrating peptides and purified Cas9 (Fig. 3.1). The *ex vivo* genome editing involves targeting the cells (somatic cells or progenitor cells) outside the body in culture followed by its reincorporation into the human body (Fig. 3.2).

Ex vivo genome editing

Zygote editing

The delivery of Cas9 (cassette, mRNA or purified protein), targeting guide sequence and HDR template (as a ssDNA or dsDNA) into the zygote or embryo can be successfully applied to edit any locus of interest in cells (Wang *et al.*, 2013). Given the permanent nature of such manipulation, the coming generation will acquire these changes possibly leading to the eradication of the disease. The

Table 3.1 List of genetic disorders treated or that can be treated with CRISPR/Cas9

Genetic disorder	Mutation	Target edited/or can be edited with CRISPR/Cas9	References
Cataracts	Multiple	*Crygc*	Wu *et al*., 2013
β-Thalassaemia	Multiple	*HBB*	Liang *et al*., 2015
Tyrosinaemia	Multiple	*Fah*	Yin *et al*., 2014
Acute myeloid leukaemia	Multiple	*MLL3*	Chen *et al*., 2014
High cholesterol level		*PCSK9*	Ding *et al*., 2014
Fanconi anaemia	c.456+4A>T	*FANCC*	Osborn *et al*., 2015
Myeloproliferative neoplasm polycythaemia vera	V617F point mutation	*JAK2*	Smith *et al*., 2015
Cystic fibrosis	Codon deletion (encoding phenylalanine)	*CFTR*	Schwank *et al*., 2013
Retinitis pigmentosa	Multiple	*RPGR*	Bassuk *et al*., 2016
Corneal dystrophy	Multiple	*TGFBI*	Usui, 2016
Duchenne muscular dystrophy	Faulty exon	*DMD*	Long *et al*., 2016
Sickle cell diseases	(A to T) in the 6th codon	*HBB*	Huang *et al*., 2015
Polycythaemia vera	V61F	*JAK2*	Smith *et al*., 2015

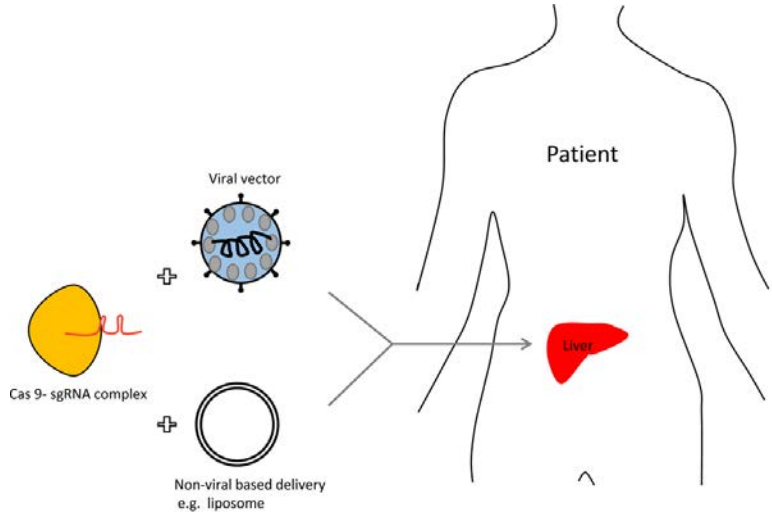

Figure 3.1 The CRISPR/Cas9 and sgRNA complex is packaged into virus or non-viral such as liquid nanoparticles to deliver it into target site, for example liver, or delivered systematically.

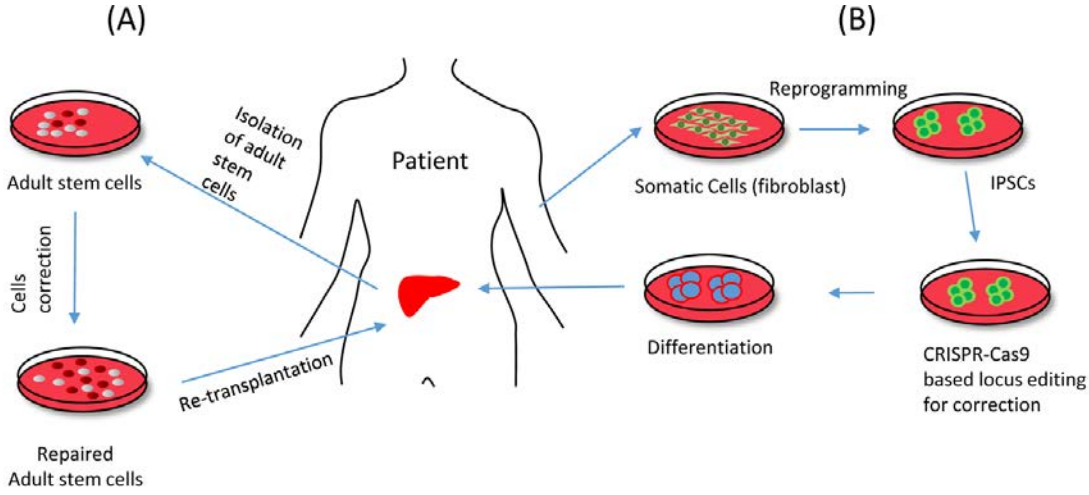

Figure 3.2 Schema of *ex vivo* genome editing. (A) adult stem cells such as haematopoietic stem cells (HSCs) and induced pluripotent stem cells (iPSCs) can be targeted for treatment followed by its reintroduction into the patient's body after clonal selection. (B) Another way is to isolate somatic cells from skin or blood such as a fibroblast, reprogramming into iPSCS, CRISPR/Cas9 mediated correction and its differentiation into HSCs followed by retransplantation into the patient's body.

first aforesaid application of CRISPR/dCas9 as a genome editing tool to treat genetic disorder was carried out by Wu *et al.* (2013) to treat an eye disorder known as cataracts. The disease is characterized by impairment in vision because of opaqueness in the eye lens. The gene responsible for this disease is *Crygc*, and in this study the researchers provided *in vitro*-transcribed Cas9 mRNA, gRNA and a template DNA containing the corrected gene of *Crygc* gene into the zygote.

In another approach the scientist treated Duchenne muscular dystrophy (DMD) by correcting the *dystrophin* gene. They applied Cas9 mRNA, gRNA and HDR template (as a ssDNA) into a mouse embryo to correct the muscular dystrophy (Long *et al.*, 2014).

In April 2015, a research group from China used the same system in non-viable human zygote to edit the β-globin gene *HBB* causing β-thalassaemia (Liang *et al.*, 2015), which created a huge controversy regarding the ethical concern in the scientific community. The scientist used the triponuclear zygote (egg fertilized by two sperms and having an extra set of chromosomes). Although they failed to achieve full-length efficiency and on-target activity, they are hopeful to optimize the system, especially for on-target specificity prior to clinical application.

The paternal genome editing allows the efficient integration of transgene into the genome compared with oocyte at sites know as quasi-random sites (Perry *et al.*, 2001; Yanagimachi, 2002). This is because, during fertilization, the decondensation (loss of nucleoprotein) makes it a better substrate for recombination. To this end, the injection of Cas9-sgRNA in decondensation phase followed by ICSI of unfertilized oocyte efficiently achieved editing of both *eGFP* and *Tyr* (Suzuki *et al.*, 2014).

In vivo genome editing

Remediation of haematological disorders

Tyrosinaemia, characterized by an elevated level of tyrosine in the blood, results in various disorders such as hereditary infantile tyrosinaemia (tyrosinaemia-I), tyrosinaemia-II and tyrosinaemia of the newborn (TTN). Tyrosinaemia-1 is also called Fanconi syndrome, with renal tubular failure in early stage of life in infants. The mutant *Fah* (fumarylacetoacetate hydrolase) gene responsible for the disease phenotype was corrected in an adult mouse by hydrodynamic delivery of a plasmid expressing Cas9 and sgRNA encoded in a plasmid rescued wild-type expression of Fah protein in 1 out of 250 cells (Yin *et al.*, 2014).

Acute myeloid leukaemia (AML) is cancer of the myeloid line of blood cells affecting adults. The incidence of disease increases with age because of deleted regions located on 7q on chromosomes. *MLL3* gene was identified as haploinsufficient tumour suppressor in AML. CRISPR/Cas9 was used to disrupt *MLL3* in $p53^{-/-}$ mouse haematopoietic stem/progenitor cells (HSPCs) which showed disease acceleration and AML development (Chen *et al.*, 2014). More recently, researchers from Wellcome Trust identified a potential gene known as *KAT2A*. CRISPR/Cas9-mediated gene disruption of this gene resulted in slower growth and better survival of leukaemic cells (Tzelepis *et al.*, 2016). A number of human malignancies are caused by mutation in more than one gene (4–5) and using conventional methods to treat such conditions is nearly impossible, however, using CRISPR/Cas9 and multiple gRNAs delivered by lentiviruses researchers modified five genes in a single haematopoietic cell (Heckl *et al.*, 2014).

Sickle cell disease (SCD) results from genetic mutations in the β-globin gene and are among the most common monogenic diseases in the world (Bauer and Orkin, 2015). Sickle cell anaemia is a genetic disease caused by homozygous mutation (A to T) in the 6th codon of the *HBB* gene which transcribes glutamic acid to valine, resulting in the production of abnormal β-globin and abnormal red blood cells production (Sun and Zhao, 2014). CRISPR/Cas9 corrected one *HBB* allele (proprotein convertase subtilisin) in high-quality iPSCs efficiently (Huang *et al.*, 2015).

The loss of function mutation in proprotein convertase subtilisin/kexin type 9 (*PCSK9*) is associated with low blood cholesterol level. Upon expression in liver this protein PCSK9 binds to the receptor and functions as an LDL receptor antagonist also degrading these receptors. This results in the lower uptake of LDL cholesterol. Using CRISPR/Cas9 the scientist disrupted *PCSK9* in mouse liver. Interestingly, the non-homologous end joining pathway (NHEJ) mediated gene disruption achieved 50% efficiency (Ding *et al.*, 2014).

Fanconi anaemia (FA) is a bone marrow failure syndrome caused by in-frame deletion in exon 4 of the *FANCC* gene, which produces congenital abnormalities and variation in chromosomal organization resulting in haematological and solid malignancies (Schifferli and Kuhne, 2015). The use of Cas9n corrected the mutation c.456+4A>T in the defective gene with higher efficiency (Osborn *et al.*, 2015).

It has been reported that 95% of polycythaemia vera (PV) is caused by a point mutation in *JAK2* (JAK2-V61F) (Levine and Gilliland, 2008) and a point mutation (AAT Z-mutation) the cause of α_1-antitrypsin (AAT deficiency) (Carrell and

Lomas, 2002). To compare the gene disruption ability of CRISPR/Cas9 and TALENs in myeloproliferative neoplasm polycythaemia vera (PV) derived iPSC to test the editing of target gene by HDR and gene disruption by NHEJ (Smith *et al.*, 2015). Cas9 induced a high frequency of indels produced as a result of DSB repair by NHE compared to that of TALENs. While the efficiency of genome editing based on HDR was comparable between CRISPR/Cas9 and TALENs.

CRISPR/Cas9 to treat movement disorders

Genetic disorders of movement or neurodegenerative disorders are caused by multiple genes and characterized by age-related accumulation of abnormal proteins causing degeneration of neurons through an unknown mechanism. Effective treatments of these diseases are of great importance. The most common neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and frontotemporal dementia (FTD). The genes coding for α-synuclein (*SNCA*) and parkin (*PRKN*) are the most important genes playing role in PD. Mostly, people with these mutations are prone to develop PD. The *SNCA* gene is crucial in PD as duplication of this locus accounts for 2% of familial cases (Lesage and Brice, 2009), because the α -synuclein protein is the main component of Lewy bodies (cytoplasmic misfolded proteins). Similarly in HD patients, on the other hand, there are aggregates or inclusions formed in an age-dependent manner by mutant huntingtin with an expanded polyQ tract (Li and Li, 2011).

There are several approaches for the treatment of these disorders based on gene silencing or gene delivery methods. These methods are in the pipeline of preclinical trials. In one study, the use of viral vector mediated Cas9 and gRNA delivery into mice brain cells using a fluorescent reporter gene to measure the effect on brain cells edited the targeted genes in neurons and astrocytes. When applied to the HD affected mice, the system efficiently reduced huntingtin (HT) accumulation (Talan, 2015). The technique led to 50% gene disruption in the test tube experiments. In another study CRISPR/Cas9 allowed knock-in designer receptors exclusively activated by designer drugs (DREADDs). The administration of clozapine-N-oxide (CNO) enables the precise regulation of human pluripotent stem cell (hPSC)-derived neurons. The transplantation of hPSC-derived human midbrain dopaminergic neurons into a Parkinson's disease mouse model rescues their motor function, and is able to be reversed or enhanced by using CNO (Chen *et al.*, 2016). These transplanted cells also caused behavioural changes in mice.

One of the most important applications of CRISPR/Cas9 is its application towards Duchenne muscular dystrophy (DMD). The disease is caused by mutation in the dystrophin gene at locus Xp21 on the short arm of the X chromosome (Ross *et al.*, 2005). Dystrophin is a protein present on the outermost layer of myofilament forming a connection between cytoskeleton and extracellular matrix. However, in its absence calcium enters into mitochondria and leads to capturing of water in the mitochondria, ultimately bursting.

Recently, CRISPR/Cas9 has been applied to shorten the dystrophin gene by removing the faulty exon in mice (Long *et al.*, 2016). The exon skipping technology with CRISPR/Cas9 can be applied to treat several other genetic diseases such as ataxia telangiectasia, congenital disorders of glycosylation and Niemann–Pick disease type C caused by errors in splicing.

Treating cystic fibrosis

Cystic fibrosis is characterized by accumulation of fluid in the gastrointestinal and pulmonary tract, leading to difficulties in breathing and recurrent infections. The gene responsible for the disease is *CFTR*, encoding a transmembrane conductance regulator. The function of this protein is to control the efflux and influx of mucus (epithelial fluid). However, the loss of function of this gene is due to deletion of three nucleotides, resulting in the loss of phenylalanine at 508th position of protein in about 70% of patients. CRISPR/Cas9-mediated gene editing allowed successful treatment of *CFTR* locus in intestinal stem cells from CF patients when transfected with a viral vector containing CRISPR components (Schwank *et al.*, 2013).

CRISPR/Cas9 to treat eye and ear diseases

Dystrophies in eyes are degenerative disorders of the eyes which, resulting from genetic heterogeneity, are characterized by symptoms such as night or colour blindness, abnormal vision and subsequent progression to complete and irreversible blindness. There are more than 120 genes associated with the development and function of eyes. Mutations in these genes are associated with different eye diseases. CRISPR may prove very fruitful to remediate these conditions because the eye is a constrained organ with relatively easy accessibility. To this end CRISPRs are also used to correct several genetic disorders of eyes, for instance retinitis pigmentosa. Retinitis pigmentosa is a serious disorder of the eye affecting approximately 1 in 4000 people in the USA. The disease is characterized by the breakdown of photoreceptor cells, resulting in gradual loss of vision, and has been recently edited successfully in iPSC for *RPGR* gene (Bassuk *et al.*, 2016). Similar application of CRISPR/Cas9 to treat the mutant gene *Crygc* has resulted in successful treatment of cataracts (Wu *et al.*, 2013).

Recently, a Japanese group has successfully applied CRISPR/Cas9 to treat transforming growth factor beta-induced (*TGFBI*) related corneal dystrophy using HDR pathway. The cultured corneal keratinocytes obtained from R124H granular dystrophy were transfected with gRNA and ssDNA HDR template *in vitro* (Usui, 2016).

The hearing loss diseases are mostly caused by mutation in a single gene, whereas few of them are caused by mutation in multiple genes (Rabionet *et al.*, 2000).

These genes are involved in different functions such as transcription factors, extracellular matrix molecules, cytoskeletal components, ion channels and transporters (cochlear molecules and hereditary deafness). The mutations in these genes are deletions, insertions, point mutations resulting in missense, nonsense or mutation of the responsible genes causing hearing disorder (http://hereditaryhearingloss.org/).

In order to develop a therapeutic strategy for a genetic disorder, understanding of the molecular mechanism and pathogenesis of the disease play a crucial role. The CRISPR/Cas9 technology platform generating a model organism or embryonic stem cells by mean of HDR or NHEJ that resembles the mutant condition can greatly help to achieve this goal.

A study has shown that a Cas9–sgRNA complex delivered by means of cationic lipid into the mouse inner hair cells *in vivo* showed efficient genome editing by knock-down of GFP signal in the Atoh1-GFP transgenic mice (Rabionet *et al.*, 2000). However, the development of a method to reduce the rapid degradation of Cas9 protein in cells and also the delivery of this complex to target the inner cells, for instance using supercharged protein to deliver Cas9-gRNA into inner cells also improving the frequency of HDR mediated gene editing to treat recessive mutation, will greatly facilitate new therapies for the treatment of genetic based deafness (Zou *et al.*, 2015).

Editing epigenome to treat cancer

The term 'epi (above)-genetics', means 'changes in the genome brought on by factors other than those related to conventional genetics', and represents a set of modifications that regulate gene expression during cell development. Epigenome editing, mainly comprising DNA methylation and histone modification, plays a crucial role in the regulation of gene expression (Jaenisch and Bird, 2003). The histone proteins which are involved in the packaging of DNA in the nucleus are modified by a number of epigenome modification including ubiquitination, phosphorylation, SUMOylation, acetylation. These reversible modification are carried out by specific enzymes (Arnaudo and Garcia, 2013). Chromatin folding and remodelling is also influenced by DNA methylation carried out by DNA methyltransferases (Jones and Takai, 2001; Reik and Walter, 2001).

Cancer is caused by changes in the epigenome, for instance in a recently reported study, the introduction of a genetic segment into the mouse genome upstream of gene p16 caused lung cancer, leukaemia or sarcomas in 27% mice compared to wild-type mice that did not induce cancer (Yu *et al.*, 2014). The gene p16 regulates cell division, and the exogenous segment used in this study was motifs from the human genome acting as a gene silencer during human development.

Methylation of cytosine plays a critical role in cancer pathogenesis. In cancer the methylation at

C is increased or decreased which in turn affects the regulatory sequences in DNA such as satellite DNA, repetitive sequences, and CpG sites. For example, mice with mutant DNA methyltransferases have low methylation in genome and developed lymphomas in T cells due to the activation of retroviral elements (Gaudet *et al.*, 2003; Howard *et al.*, 2008). However, in some human tumour conditions it is the overactivity of methyltransferases causing rapid proliferation of malignant cells (Issa, 2004).

Histone proteins are small proteins that work as a template to wrap the DNA to form the nucleosome. During the formation of the nucleosome the amino acids in the tail region (N-terminal) are exposed to enzyme activities responsible for epigenetic modification such as acetylation and phosphorylation, ubiquitination and methylation, and these modifications attribute to the tight or relaxed conformation of chromatin (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The acetylation and phosphorylation are responsible for open chromatin structure as they represent the activation mark. The methylation may acts as either an activating mark (Strahl and Allis, 2000) or repressive mark (Czermin *et al.*, 2002; Müller *et al.*, 2002).

The exploration of the epigenome will provide a comprehensive understanding of its structure, function and association with diseases. To do so, dead Cas9 (dCas9) fused with a repressor, activator or modifier has been used. The fusion of dead Cas9 (double mutant Cas9 with no cleavage but binding ability) with Krüppel-associated box (KRAB) achieved suppression of targeted H2S enhancer region. The degree of genome-wide specificity and heterochromatin formation was not explored in this study. H2S enhancer, which enhances the expression of multiple globin genes using dCas9-KRAB trimethylation (H3K9me3) as the enhancer, was reported (Thakore *et al.*, 2015). The *Tyr* gene encoding for tyrosinase is a principal enzyme involved in the melanin biosynthesis pathway (Lavado and Montoliu, 2006; Olivares and Solano, 2009). Mutations in *Tyr* results in low melanin synthesis resulting in a genetic condition known as albinism. Using CRISPR/Cas9 guided by two gRNAs approach, a group of researchers deleted a 5′ region present ~12 kb 5′ region upstream of the mouse *Tyr* locus (Seruggia *et al.*, 2015). The resultant mice lacking the DNA sequence showed a clear phenotype. Histone acetyltransferase (HAT) catalytic core of the human acetyltransferase p300 is involved in a number of cellular processes (Chen and Li, 2011; Ogryzko *et al.*, 1996). Using dCas9 fused with catalytic histone acetyltransferase, acetylation of histone H3 lysine 27 at its target site was reported which significantly resulted in the regulation of epigenome and downstream gene expression (Hilton *et al.*, 2015).

The non-coding RNA (ncRNA) species, for instance micro-RNA (miRNA) AND short interfering RNA (siRNA), has been shown to regulate the epigenetics, thus regulating vital biological processes such as growth and development (Falahi *et al.*, 2015). In the prostate more than 100 regions are associated with the disease progression and recently more than 45 genes coding for non-coding RNAs are involved in the disease (Guo *et al.*, 2016). The targeting of these non-coding RNAs, especially miRNAs involved in cancer progression and development, could prove fruitful for cancer treatment.

CRISPR/Cas9 to generate disease models

The application of mouse models in regenerative medicine has played a tremendous role. The ability of Cas9 to edit genes by bringing point mutations or translocation at any locus in diverse cell lines has allowed the study of the function of many of these genes. Moreover, embryonic genome editing has paved the way to generate animal models to study diseases (Fig. 3.3). The benefit of the generation of these models is their resemblance with humans, thus allowing better understanding of the pathogenesis and progression of diseases, validating drug target sites and effective treatments, e.g. generation of a monkey model deficient in dystrophin gene has been shown to possess the same symptoms as a patient (Chen *et al.*, 2015). Similarly the system was recently applied to develop a pig model of Parkinson's disease by editing the responsible genes (Wang *et al.*, 2016a; Zhou *et al.*, 2015).

Huntington's disease (HD) is an age-dependent neuronal degenerative disorder caused by the abnormal repetition of trinucleotides (coding for glutamine residues) in the N-terminal of huntingtin (*htt*) gene (Gusella *et al.*, 1993). However, using nuclear transfer technology scientists generated a HD pig model expressing N-terminal mutant *htt* gene (Yang *et al.*, 2010). Using Cas9-gRNAs

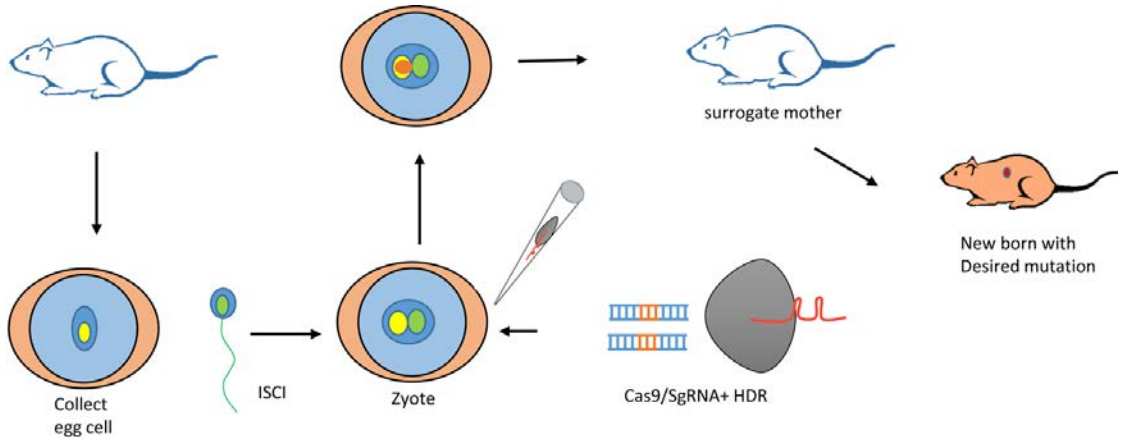

Figure 3.3 Representation of generating a transgenic mouse model using CRISPR mediated genome editing. The egg is collected from the donor, fertilized *in vitro*, and the zygote is engineered with CRISPR/Cas9 and implanted into surrogate mother. Post gestation mutant mice with desired mutations are born.

provided with exogenous desired mutant donor DNA fragments may be used to generate a model organism expressing the mutant gene.

The main hurdle to generating transgenic animal models is genetic mosaicism, arising due to the low rate and efficiency of Cas9 in inducing mutations. This may reflect the repression in translation of Cas9 in the zygote after microinjection of Cas9 mRNA and gRNA, but more likely the translation of Cas9 mRNA transcript is delayed after the first cell division (Oh *et al.*, 2000). In consistent to the previous discussion, the results of NHEJ repaired pathway introduce indels resulting in genetic mosaicism in transgenic rice. To this end efforts are made to supply single-cell fertilized embryos with purified Cas9 and sgRNA (Gratz *et al.*, 2013).

Genome editing of viruses

Hepatitis B virus (HBV) is a partially double-stranded DNA virus belonging to the Hepadnaviridae family. The chronic infection of HBV causes liver cirrhosis and carcinoma. Upon entry into the cell, the partially double-stranded DNA is converted into fully double-stranded DNA, i.e. covalently closed circular DNA (cccDNA) by viral polymerase. This cccDNA is highly stable in the cell and acts as a template for viral replication, thus making the removal of the virus challenging. Using Cas9 the researchers edited the viral genome by hydronamic injection of Cas9 and gRNA into the mouse tail that was already transfected with the HBV expression plasmid (Lin *et al.*, 2014a). Interestingly, the authors observed the cleavage of the viral vector and reduced surface antigen in the serum.

In a study carried out by Ebina *et al.* (2013), CRISPR/Cas9 was used to edit the HIV-integrated proviral DNA. Using gRNA to target HIV-1 long terminal repeat sequence at the 5′ and 3′ termini resulted in the subsequent suppression of the gene driven by LTR that in turn resulted in the inactivation of HIV replication in latently infected cells. This study also demonstrated the removal of viral DNA from the host chromosome. The use of dual gRNAs was shown effective to remove viral DNA fragment of hepatitis B (Wang *et al.*, 2015). These results indicate that the CRISPR/Cas9 technology can serve as a potential tool for clinical applications to cure infectious diseases. The use of this tool has been found to be effective in the clearance of latent viral DNA of hepatitis B virus, HIV and herpes virus (Hu *et al.*, 2014; Lin *et al.*, 2014a; Wang and Quake, 2014).

In a recent application of Cas9 to mutate HIV-1 integrated into cellular DNA, it was found that indel mutations resulted as a result of repair of Cas9-mediated DSB and impeded viral replication; however, some viruses escaped from CRISPR immunity (Wang *et al.*, 2016b). These results show that, although some of these indel mutations disrupt the recognition fidelity of Cas9, they are not lethal to the virus. Multiple targeting of viral DNA with CRISPR/Cas9 might help in effective

antiviral therapy. The application of CRISPR/Cas9 to inactivate the viral genome has been carried out with a number of viruses including hepatitis B (Dong *et al.*, 2015; Karimova *et al.*, 2015; Lin *et al.*, 2014a; Ramanan *et al.*, 2015; Zhen *et al.*, 2015), Epstein–Barr (Wang and Quake, 2014; Yuen *et al.*, 2015), vaccina (Yuan *et al.*, 2015), adenovirus (Bi *et al.*, 2014), herpes simplex type 1 (Bi *et al.*, 2014) and human papillomavirus (Kennedy *et al.*, 2015). These applications proved that the CRISPR/Cas9 technology holds the capacity to be used as a potential tool for clinical applications to cure viral-based infectious diseases.

How to address the challenges associated with the application of CRISPR/Cas9 technology?

Programmable nuclease are guided by a stretch of guide sequence to target gene of interest, however, sometimes due to homology with secondary genomic sites, these nucleases also cause mutation in other sites known as off-target mutations. These mutations in some cases might have deleterious effects, for instance when applied in human clinical studies. The reason for such poor specificity is the fact that Cas9 uses a short stretch of 20 bp as a guide sequence in which 8–12 bp are highly crucial for targeting (Cong *et al.*, 2013). More possibly the requirement of 5′-NGG-3′5-′NAG-3′ as a PAM (protospacer adjacent motif) sequence also affects the recognition fidelity of Cas9. In order to overcome these problems there are various strategies developed by scientists to ameliorate the on-target specificity of Cas9, reviewed in recent comprehensive reviews (Jamal *et al.*, 2015; Tsai and Joung, 2016).

The development of safe and efficient delivery methods of Cas9–sgRNA complex to allow reduced toxicities and side effects is of paramount importance. Moreover, attempts should be made to avoid the random integration of donor DNA. The methods developed in last two decades can be practically applied for the delivery of Cas9-gRNA into cells (Kay, 2011; Mingozzi and High, 2011). Recently used adeno-associated virus shuttle vector is the optimum choice for the cargo method because of low cytotoxicity and immunogenicity (Ran *et al.*, 2015).

The fate of genome editing *in vivo* is different from *ex vivo*. The benefit of genome editing *ex vivo* is high chances to select cells with the corrected genotype without off-target mutation, expansion and its reintroduction into the patient's body. But the demerit of this approach is that during clonal expansion the cell may acquire unwanted genome alterations, and especially the induced pluripotent cells are more vulnerable to accumulate mutation and variation during expansion (Gore *et al.*, 2011; Ji *et al.*, 2012). In order to gain the genetic stability and avoid these unwanted mutations the culturing of stem cells in three-dimensional organoid cultures is beneficial (Huch *et al.*, 2015).

The low frequency of HDR-mediated genome editing *in vivo* compared with NHEJ is another hurdle associated with the therapeutic application of CRISPR/Cas9. During the cell cycle, NHEJ appears to be dominant during the S and G2 phases (Heyer *et al.*, 2010); HDR pathways, in contrast, are more prominent in the G1 and M phases (BP1, BRCA1 and the choice between recombination and end joining at DNA double-strand breaks). Cell cycle synchronization (Lin *et al.*, 2014b) and the use of an inhibitor (Chu *et al.*, 2015; Maruyama *et al.*, 2015) have been found to achieve a high frequency of HDR-mediated genome editing. The use of Cas9 nickase (Cas9n) with paired gRNAs has been actively used to promote HDR frequency (Ran *et al.*, 2013).

Conclusion and future directions

CRISPR/Cas9 is entering the era when it can revert the deleterious mutations causing genetic diseases at the DNA level. The scope is not limited to monogenic defects, where the strategy of using CRISPR/Cas9 is very straightforward, but extends to polygenic diseases, which can now be handled even at single-cell level. The power of CRISPR/Cas9 is no doubt very great, but its target-oriented use requires highly sophisticated clinical assays to e established to avoid any unwanted side-effects. The future of CRISPR/Cas9 genome editing is promising, and several laboratories are presently exploring its applications in wide areas of genome biology and personalized medicine.

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Conflict of interest

The authors disclose no conflict of interest.

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